IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
APPLICATION FOR LETTERS PATENT

TO ALL WHOM IT MAY CONCERN:

BE IT KNOWN THAT

Patricia Guerry, Edward Burg, Lanfong H. Lee and Trevor J. Trust

Citizens of the United States of America and residents of Rockville, MD; Washington, D.C.; Silver Spring, MD and Sherborn, MA, respectively have invented new and useful improvements in

A RECOMBINANT POLYPEPTIDE FOR USE IN THE MANUFACTURE OF VACCINES AGAINST CAMPYLOBACTER INDUCED DIARRHEA AND TO REDUCE COLONIZATION

of which the following is a specification and for which a Patent is requested:

SPECIFICATION

Cross Reference to Related Patents and Applications

This application is related to the Provisional Application for Patent entitled, "A Recombinant Polypeptide For Use In The Manufacture of Vaccines Against Campylobacter Induced Diarrhea filed November 12, 1998 by the inventors Patricia Guerry, Edward Burg, Lanfong H. Lee, and Trevor J. Trust, and having serial no. 60/108,114, and is entitled to the benefit of the November 12, 1998 filing date for the matter disclosed therein. That Provisional Application for Patent is incorporated herein by reference.

Background of the Invention

Field of the Invention

The present invention relates to a construct of a recombinant DNA containing a fragment

of a bacterial gene and the expression of the constructs for use as a vaccine or a component of a vaccine. Moreover, the invention relates to a recombinant protein comprising the maltose binding protein (MBP) of Escherichia coli fused to amino acids 5-337 of the FlaA flagellin of *Campylobacter coli VC167.*

Description of the Prior Art

The genus *Campylobacter* are gram-negative, curved, spiral or S-shaped or, in some cases, coccoid, bacteria. *Campylobacter* have a single polar, unsheathed flagellum at one or both ends which imparts a characteristic darting or cork-screw motility. *Campylobacter* are a major cause of gastroenteritis in both developed and developing countries (1,2). The major enteric pathogens in the genus are *C. jejuni* and *C. coli.* All can be normally present in the gastrointestinal tract of domestic and wild animals which act as a major reservoir for infection in humans. Human infection with *Campylobacter* occurs via:

1) animal to human contact especially farm animals such as poultry;
2) human to human transmission especially from infected children;
3) food contamination;
4) water contaminated with excreta from animals.

Campylobacter infection can produce both an inflammatory diarrhea and a non-inflammatory diarrhea. The infection is more likely to be of the non-inflammatory type, without fever or bloody diarrhea. However, severe bloody diarrhea resembling bacillary dysentery can occur and frequently is seen in travelers to developing countries.

Although *Campylobacter* diarrhea is treatable with antibiotics, an effective vaccine against the organism is much preferred. This is especially true for travelers to regions where the disease is endemic and for use by developing nations where antibiotics are not always available or where their cost prohibits their use by the general population. There are, however, no currently licensed vaccines available against these organisms.

An important, possible contraindication of whole cell Campylobacter vaccine is the potential for development of Guillain-Barre Syndrome (GBS) (3), a post-infectious polyneuropathy, in vaccinated individuals. There are several reports indicating that prior infection with *C. jejuni* can result in acquisition of immunity (10,11). However, development of vaccines has been hampered by a lack of understanding of the basic virulence mechanisms and by the antigenic complexity of these organisms. For example, the serotyping scheme developed by Lior (12) is based on heat labile (HL) antigens and has over 100 recognized serogroups. The heat stable serotyping scheme of Penner (41), which is thought to be based on lippolysaccharides (LPS), has over 70 serotypes. The LPS cores of many serotypes have been shown to contain sialic acid in structures which resemble human gangliosides (13). This molecular mimicry has been implicated in development of autoantibodies leading to GBS, although the specific structure or structures which enable a given campylobacter strain to cause GBS are not clear. Strain 81-176 belongs to Serogroup O:23,36. Strains of O:23 and O:36 have been shown to contain ganglioside-like structures in their lipopolysaccharides. Although some O serotypes of *C. jejuni* are implicated with inducing GBS, O serotyping alone is insufficient in determining the potential

for a given strain to induce GBS. Also, there is insufficient information to determine definitively the ability of any Campylobacter strain to lead to development of GBS.

A formalin fixed whole cell vaccine of *C. jejuni* 81-176 adjuvanted with mutant E.coli heat labile enterotoxin (LT $_{R192G}$; 12) is currently in human testing (14,15). This formulation appears to offer protection against homologous challenge in animal models (23,9), but the ability to protect against multiple serotypes of *C. jejuni* remains to be determined. Moreover, given the lack of understanding about the pathogenesis of *Campylobacter* associated GBS, there are concerns about use of whole cell preparations of *campylobacter* as vaccines. This concern becomes more compelling if multiple strains, which are less well characterized than 81-176, were to be combined in order to generate broad cross-serotype specific protection. An alternate approach would be to utilize a single *campylobacter* protein, either as a recombinant subunit vaccine or expressed in a carrier vaccine strain, to elicit protection against multiple serotypes of *Campylobacter*. Therefore, there exists in the current state-of-the-art, the question whether specific *Campylobacter* strains, used in whole-cell vaccines or whole-cell vaccine candidates, could potentiate GBS and therefore be safe for vaccine use. One candidate for inclusion into such vaccines is flagellin.

Flagellin is a component of flagella, which provides swimming motility on many bacterial species including *Campylobacter*. Flagellin is the immunodominant antigen recognized during human and experimental animal infections (16,17,18) with *Campylobacter*. The structure of flagellin has been determined experimentally using the *Campylobacter coli* strain VC167 as a

model. The flagella of this organism is complex, composed of multiple species of flagellin subunits, FlaA and FlaB (4-6). The FlaA and FlaB subunits are encoded by two genes, flaA and flaB, that are located adjacent to one another in a tandem orientation (figure 1). The expression of these genes is concomitant and unit length rather than polycistronic. The flaA flagellin gene, which encodes the major flagellin subunit in the complex flagellar filament, has been divided into five regions (5) based on restriction enzyme mapping. Regions I - III encode the most highly conserved regions of the protein among different Campylobacter flagellin genes and are also the most immunodominant region of the protein (7).

Because of the potentially harmful effects of using whole-cell *Campylobacter* vaccines, it was concluded that an effective vaccine against this organism was needed that, at the same time, did not induce the deleterious autoimmune responses.

SUMMARY OF THE INVENTION

Accordingly, an object of this invention is a recombinant construct and expressed protein possessing highly immunogenic regions of the flagellar subunit but which did not contain antigenic moieties which induce GBS.

Another object of the invention is a recombinant protein encoding a portion of the flaA flagellin gene of *Campylobacter coli* VC167 encoded by nucleotides 13-1,015 corresponding to amino acid residues 5-338 (8).

Yet another object of the invention is a product that can be expressed in different host backgrounds of bacterial strains belonging to the family *Enterobacteriaceae* for use in different vaccine formulations against Campylobacter.

An additional object of this invention is the induction of a host immune response by purified recombinant flagellin expressed in *E. coli*.

A further object is the cross-reactivity of antibodies, induced by purified recombinant flagellin expressed in *E. coli*, with flagellins from *C. coli* (VC167) and other strains of *Campylobacter* spp.

Another object is that the purified recombinant flagellin expressed in *E. coli* is capable of protecting animals from disease and intestinal colonization by a heterologous strain, *Campylobacter jejuni* 81-176.

A still further object is the use of the construct in vaccine preparations against diarrhea yet reducing the potential for GBS.

Another object is the use of the contstruct in vaccine preparations in order to reduce intestinal colonization.

These and other objects of the invention are accomplished by a recombinant DNA construct encoding the immunodominant region (regions I through III) of flagellin from *Campylobacter* spp. for use as a component of a vaccine against *Campylobacter* disease.

BRIEF DESCRIPTION OF THE DRAWINGS

A more complete appreciation of the invention will be readily obtained by reference to the following Description of the Preferred Embodiments and the accompanying drawings in which like numerals in different figures represent the same structures or elements. The representations in each of the figures is diagrammatic and no attempt is made to indicate actual scales or precise ratios. Proportional relationships are shown as approximations.

Fig. 1 is a schematic representation of the flaA and flaB genes *of C. coli*, VC167, which encodes the major flagellin subunits in the flagellar filament.

Figure 2 is a schematic representation of the regions I through V of flaA. The size, in nucleotide base pairs, of each region is shown. The immunodominant regions are regions I, II and III.

Figure 3 is a comparison of the amino acid sequences ~~of flagellins flaA of *Campylobacter coli* VC167T2 and~~ *Campylobacter jejuni* 81-176.

Figure 4 is a graph of the experiment evaluating the ability of MBP-flaA to protect against colonization following oral feeding of mice.

DETAILED DESCRIPTION

Currently, no efficacious vaccine exists for *Campylobacter* disease. The potential for GBS complicates using attenuated strains of *Campylobacter* as a vaccine. It is critical therefore, that a vaccine be produced that is both capable of eliciting a vigorous protective immune response but does not lead to GBS.

Sub-unit vaccines hold a great deal of promise in fulfilling both these important criteria. One of the most highly immunogenic exposed regions of *Campylobacter* is the flagellin proteins, in particular the products of the genes flaA and flaB. The flaA gene of *Campylobacter* has been divided into five regions (5) based on restriction enzyme maps. This region of the flaA gene contains both highly conserved and variable regions among *Campylobacter* species. The preferred embodiment of this invention encompasses the gene sequence and expression from 13 through

1,015 base pairs of the flaA gene of *C. coli* (6), covering regions I, II and III as shown in figure 2, and segments within this region. This gene sequence is used to produce a recombinant *Campylobacter* polypeptide, which if introduced into a host is capable of producing an immunological response. The FlaA flagellin is the major protein subunit comprising the flagella filament or locomotory organelle of *Campylobacter spp.*

Ins B

~~Region I of the flaA gene represents the highly~~ conserved N terminal region, and regions II and III represent two regions which are more variable among different sequenced flagellin genes. Regions II and III are not, however, as variable as region IV. The construct was made by amplifying the regions I, II and III using the primer flaA-11 (5'ACCAATATTAACACAAATGTTGCAGCA3') (Seq. ID no. 3) and flaA-2 (5'TTATCTAGACTAATCTCTACCATCATTTTTAAC3') (Seq. ID no. 4). The PCR product is digested with the appropriate restriction enzymes in order to insert the product into an expression vector. Any plasmid expression vector, e.g. pET (Novogen, Madison Wisconsin) or pMal (New England Biolabs, Beverly, MA) and viral expression vectors (e.g. adenovirus, M13, herpesvirus, vaccinia, baculovirus, etc) expression systems can be used as long as the polypeptide is able to be expressed. The preferred expression system is the pMal-c2 vector (New England Biolabs, Beverly, MA). For insertion into this system the PCR product is digested with SspI and XbaI, purified by agarose gel electrophoresis, and cloned in a commercially available plasmid vector, pMal-p2 or pMal-c2 (New England Biolabs, Beverly, MA) which had been digested with XmnI and XbaI. This vector allows for fusion of the fifth codon of the flaA gene to an *Escherichia coli* gene encoding maltose binding protein (MBP). The MBP-FlaA fusion is transcriptionally

regulated by a P_{tac} promotor and is induced by growth ~~in isopropyl~~thiogalactoside (IPTG). Several transformants of *E. coli* DH5~~-alpha~~, containing plasmids with the appropriate size insert, were sequenced ~~with the~~ MalE primer (New England Biolabs). One plasmid with the expected fusion-protein in the correct ~~reading frame to MalE, termed pEB11-2, was purified~~.

The MBP-FlaA fusion protein was purified on the basis of the ability of the MBP portion of the molecule to bind to an amylose affinity column.

Having described the invention, the following examples are given to illustrate specific applications of the invention including the best mode now known to perform the invention. These specific examples are not intended to limit the scope of the invention described in this application.

Example 1

Express~~ion of recombinant~~ flaA gene in pMal-p2/c2 plasmid.

Purification:

The purification scheme of the construct was as recommended by the commercial supplier of pMal plasmid (New England Biolabs). DH5 alpha containing the flagellin-MBP fusion was grown overnight in 100 ml Rich medium (10g tryptone, 5 g yeast extract, 5 g NaCl, 2 g glucose/liter) supplemented with 100 microgram/ml, and used to inoculate a fresh 1 liter of the same medium. This culture was grown with shaking at 37°C to OD_{600} 0.5 and IPTG (isopropyl-B-thiogalactoside) was added to a final concentration of 0.3mM. Cells were grown for an additional 2 hours and harvested by centrifugation. Cells were resuspended in 10 ml column buffer (20mM Tris-Cl, 200 mM NaCl, 1 mM EDTA). The cells were frozen at -20°C overnight,

thawed on ice, and sonicated in short pulses for 2 min using a Branson sonicator (Branson, Danbury, CT). The sonicated solution was centrifuged for 30 min at 9000g in a Sorvall RC5-B centrifuge, and the supernatant was diluted 1:5 with column buffer and loaded onto a 2.5 X 10 cm glass column packed with 15 ml of amylose resin (New England Biolab) at a flow rate of 1 ml/min. The column was washed with 12 volumes of column buffer and the fusion protein was eluted with column buffer containing 10 mM maltose. Protein containing fractions (as determined by BioRad protein assay) were pooled, concentrated using a vacuum concentrator, and stored in aliquots at -20°C.

Characterization:

The apparent M_r of the protein produced by DH5 alpha (pEB11-2) after IPTG induction was approximately 80,000, as determined by SDS-PAGE electrophoresis. This is consistent with the predicted M_r of 79,687 for the fusion protein, which includes M_r 34,678 of FlaA and M_r 45,009 from MBP. The fusion protein was immunoreactive with two hyperimmune rabbit antiserum made against flagellin purified from two strains of *Campylobacter*: antiserum E288 which was made against purified flagellin from the homologous strain, *C. coli* VC167 (8) and antiserum SML2 which was made against purified flagellin from *C. jejuni* strain VC74 (9).

Antibody from natural infections was shown to recognize the native protein better than the recombinant protein as shown in Table 2.

Electrophoresis and Western Blotting. SDS-PAGE was performed with a mini-slab gel apparatus (Pharmacia, Piscataway, NJ) by the method of Laemmli (19). Proteins samples

solubilized in sample buffer (21) were separated in 12.5% acrylamide (150V) and either stained with Coomasie brilliant blue or transferred to nitrocellulose for immunological detection (20). Rabbit hyperimmune sera E288 against SDS-denatured VC167 T2 flagellin has been described previously (7). Immunodetection was described in Power et al. (7). The secondary antibody for rabbit antisera was alkaline phosphatase tagged goat anti-rabbit IgG (Caltag, Burlingame, CA) used at a final dilution of 1:5000; the secondary antibody for ferret antisera was horse radish peroxidase (HRP) labelled goat anti-ferret IgG (Kirkegaard and Perry, Gaithersburg, MD) used at a dilution of 1:500. Alkaline phosphatase tagged antibodies were developed with NBT-BCIP (Nitro Blue Tetrazolium, 5-bromo-4chloro-3-indolylphosphate; Promega, Madison, WI) and peroxidase labelled antibodies were developed with TMB (3,3',5,5' tetramethyl benzidine; Sigma, St. Louis, MO).

Purification of flagellin. Flagellins were purified from *Campylobacter* spp. by the method of Power et al. (37).

Immune animal sera. Immune ferret sera were obtained from a collection of sera at NMRC/FDA from experiments in which ferrets were fed either VC167 T2 or 81-176 **and** subsequently developed diarrhea (21,22) as shown in Table 2.

Immune human sera. Immune human sera from volunteers fed 81-176 was the generous gift of David Tribble of NMRC.

Hyperimmune rabbit antiserum. Antiserum against the MBP-FlaA in 1ml Freund's incomplete adjuvant 2 weeks later. The animal was exsanguinated two weeks after the second

injection and the resulting antiserum was called LL1.

ELISA. MaxiSorp 96-well immunoplates were coated with MBP-FlaA or flagellins purified from campylobacters (0.3 micrograms/ml, 100 ul/well). ELISA's were performed as previously described (23).

DNA sequence analysis of the flaA gene of *C. jejuni* 81-176. The flaA gene of *C. jejuni* 81-176 has been cloned previously and the 5'end partially sequenced (24). The intact flaA gene of 81-176 as cloned in pK2-32 (24) was sequenced in order to determine the extend of similarity between the VC167 FlaA protein and that of 81-176 which would represent the challenge strain in protection experiments. The results indicate that the flaA gene of 81-176 encodes a protein of 574 amino acids with a predicted M_r of 59,240. Overall the two proteins are 92% identical and 94% similar. VC167T2 flagellin has 573 amino acids with a predicted M_r of 59,047. The region of the VC167 T2 FlaA which is included in the MBP-FlaA recombinant protein is amino acids 5-337. The region includes those amino acids which appeared to be the most immunogenic by memeotope analysis. The VC167 and 81-176 flagellins are 98.1% identical and 98.7% similar in this region. The homology is lowest between amino acids 382 and 471. In this region, which includes an additional amino acid in the 81-176 protein, the two flagellins are 73% identical and 84% similar. Comparison of the region of VC167 T2 flagellin in the MBP-FlaA fusion protein with 11 other *C. jejuni* flagellins revealed a range of 82-90% similarity. See Figure 3.

Example 2

Use of recombinant flagellin to induce an immune response in rabbits.

Antibodies against the recombinant MBP-FlaA fusion protein are generated in rabbits by injecting rabbits intramuscularly with two (2) doses of 80 micrograms/rabbit for each dose of MBA-FlaA, the first with Freund's complete adjuvant and the second with freund's incomplete adjuvant, two (2) weeks apart. This anti-MBP-FlaA anti-serum reacted with the MBP-FlaA, MBP alone, and with flagellins isolated from both *C. coli* strain VC167 and the *C. jejuni* strain 81-176.

Example 3

Use of recombinant flaA construction for production of subunit vaccine in the protection of mice against Campylobacter disease with or without *E. coli* enterotoxin (LT) as adjuvant.

Campylobacter spp. are primarily pathogens of primates and does not cause diarrheal disease when fed to mice. However, when infected intranasally with *Campylobacter* mice develop a lung infection, often with bacteremia, and swallow enough bacteria to become colonized in their gastrointestinal tract (9). This model has been used previously to evaluate vaccines against *campylobacter*.

BALB/c mice (6-8 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in laminar flow cages for a minimum of 8 days before being used in experiments. Standard laboratory chow and water were provided ad libitum. Mice were anaesthetized with methoxyflurane (Metofane, Pitman-Moore, Mundelein IL) and immunized intranasally with 30-35

micrograms/l of fusion protein using a micropipet. The doses used were 0, 3, 6, 12, 25 or 50 micrograms of fusion protein in PBS, either with or without 5 ug of heat labile *E. coli* enterotoxin (LT) as adjuvant. A second dose was administered 8 days after the first vaccination. Intestinal lavage was collected 15 days after the first vaccination and blood was collected 29 days after the first vaccination. The mice were challenged intranasally with *C. jejuni* 81-176 (2×10^9 /mouse) 34 days after the first vaccination, and the animals were monitored for sickness and death for 5 days. An illness was determined by assigning a score of 0 (healthy), 1 (ill as determined by a hunched back, ruffled fur, lethargy) or 2 (death) for each mouse daily. For each observation day the total score within each group was divided by the number of mice observed to yield the daily index. Fecal pellets were collected daily for 10-14 days after challenge, homogenized in PBS, and plated onto a campylobacter selective agar (CVA; Remel, Lenexa, KA). Putative campylobacter colonies were confirmed by morphology and oxidase reactions. Vaccine efficacy was calculated as (rate for control mice) - (rate for vaccinated mice)/(rate for control mice) X 100.

The effect of the vaccine on disease symptoms and colonization are summarized in Table 1. The mean disease index on day 5 of mice which received no vaccine was 0.924 and for mice which received only LT was 0.859. The disease index of mice receiving MBP-FlaA without adjuvant decreased as the dose of vaccine increased up to 50 micrograms (disease index = 0.371, reflecting 55.3% efficacy). In all cases the addition of LT decreased the disease index compared to the corresponding dose of MBP-FlaA without LT. A dose of 50 micrograms of MBP-FlaA achieved an 81% efficacy in protection against disease. In previous experiments, infection of mice

with live 81-176 resulted in 71% efficacy against disease symptoms following a second challenge with the same.

There was no effect on the numbers of mice colonized with 81-176 at any dose of MBP-FlaA without LT, except at the highest dose (50 micrograms) which showed 47.6% efficacy against colonization. Similarly, mice receiving 3 micrograms, 6 micrograms or 12 micrograms of MBP-FlaA + LT showed no significant reduction in colonization. However, doses of 25 micrograms or 50 micrograms of MBP-FlaA + LT resulted in 61% and 84% efficacy against colonization, respectively. In previous experiments, infection of mice with live 81-176 resulted in 91% efficacy against colonization following a second challenge with the same strain.

The FlaA component of the MBP-FlaA fusion protein is capable of eliciting an immune response which can give significant protection against disease symptoms by a heterologous strain of *Campylobacter* as measured in this animal model, as well as against colonization of the intestine. Protection against colonization would preclude development of diarrheal disease. The regions of the FlaA protein from *C. coli* VC167 which are conserved in the portion of the protein used in these experiments are apparently sufficient to elicit protection against heterologous challenge by *C. jejuni* 81-176. This recombinant construction could be (1) used as a fusion protein, as in the example given here, to MBP, (2) be purified via another recombinant construction as a protein of approximate M_r of 35,000, expressed in another expression system, such as a histidine-tag vector system, or (3) be expressed under control of an appropriate promoter in a carrier vaccine strain such as *Salmonella* or *Shigella* attenuated live vaccines, for use as a bivalent vaccine.

Genbank accession number. The DNA sequence of the 81-176 flaA gene has been deposited in Genbank under accession number AF15052.

Example 4

Evaluation of immunogenicity and efficacy of the MBP-FlaA protein against heterologous challenge in the mouse intranasal model.

Mice were immunized intranasally with 2 doses of 3-50 micrograms of MBP-FlaA with or without 5 micrograms of LT_{R192G} as adjuvant. Table 3 shows the intestinal IgA and serum IgG response to MBP-FlaA as measured by ELISA. The full range of MBP-FlaA doses elicited significant antigen-specific serum IgG responses in vaccinated animals and these responses were enhanced by adjuvant use, with the exception of the highest dose (50 micrograms). In contrast, stimulation of FlaA-specific intestinal secretory IgA (sIgA) responses required immunization with higher does of MBP-FlaA ($\geq$25 micrograms) or co-administration of lower doses with adjuvant. When given with the adjuvant, as little as 3 micrograms of the MBP-FlaA protein was capable of stimulating a significant antigen-specific sIgA response in immunized animals. In addition, the magnitude of intestinal sIgA responses to the recombinant protein were significantly enhanced in those animals receiving the adjuvanted protein compared to those given *MBP-FlaA* alone, with the exception of the highest dose.

The mice were challnged intranasally with *C. jejuni* 81-176 (2X 10^9 bacteria/mouse) 26 days after the second immunization. The effects of the vaccine on disease symptoms and colonization on day 7 are summarized in Table 3. The mean disease index of mice which had

received no vaccine or LT_{R192G} alone was 0.92 and 0.85, respectively. The disease index of mice receiving MBP-FlaA without adjuvant decreased as the dose of vaccine increased up to 50 μg (disease index = 0.37, reflecting 55.3% efficacy), although the results were not statistically significant. In all cases, except the 3 microgram dose, the addition of LT_{R192G} decreased the disease index compared to the corresponding dose of MBP-FlaA without LT_{R192G}. A dose of 50 microgram of MBP-FlaA+ LT_{R192G} achieved 81.1% efficacy in protection against disease (P<0.0001). In previous experiments, when mice which had been infected intranasally with live 81-176 were rechallenged 26 days later with the same strain, there was 71% efficacy against disease symptoms.

There was no effect on the numbers of mice colonized with 81-176 at any dose of *MBP-FlaA* without LT, except at the highest dose (50 micrograms) which showed 47.6% efficacy in protecting against colonization. Similarly, mice receiving the lower doses of *MBP-FlaA* + *LT_{R192G}* showed little to no reduction in colonization. However, a dose of 50 micrograms *MBP-FlaA* + *LT_{R192G}* resulted in 84.1% efficacy against colonization (P<0.05). In previous experiments using this model, infection of mice with live 81-176 resulted in 91% efficacy against colonization following a second challenge with the same strain.

Example 5

Evaluation of the ability of MBP-FlaA to protect against colonization following oral feeding of mice.

To better examine the ability of MBP-FlaA to protect against intestinal colonization,

additional mice were vaccinated with two doses each of 50 micrograms *MBP-FlaA* with and without 5 micrograms LT_{R192G} adjuvant, 8 days apart. Twenty-six days after the second immunization, groups of 7-8 mice were challenged orally with 3 different doses of 81-176: $8x10^{10}$, $8x10^{9}$ and $8x\ 10^{8}$. Control animals immunized with either PBS (open triangles) or LT_{R192G} (closed triangles) alone were colonized throughout the course of the experiment regardless of challenge dose. These results are shown in Fig. 4A for the highest challenge group only. Animals immunized with *MBP-FlaA* alone showed an apparent transient and insignificant reduction in total numbers colonized at days 5 and 6 (71.4% of the animals were culture positive) in the high dose challenge group only Fig. 4A. (open circles) However, on day 7 100% of the mice immunized with *MBP-FlaA* alone were colonized. When animals immunized with *MBP-FlaA* + LT_{R192G} were challenged with 8×10^{10} organisms, there was a marked difference between the controls at days 5-7, with only 40% of the animals being colonized on days 5 and 6 ($P <$ 0.05), and 20% colonized on day 7 (P <0.001; Fig. 4A (closed circles). This corresponds to 55.2% efficacy for days 5 and 6, and 71.4% efficacy on day 7. The efficacy improved when the animals were challenged with 8×10^{9} bacteria (Fig. 4B). In this case, a significant difference between *MBP-FlaA* + LT_{R192G} (closed circles) and *MBP-FlaA* alone (open circles) and control groups was apparent by day 5, with the *MBP-FlaA* + LT_{R192G} vaccine giving 55.1% efficacy (P <0.05). By day 7 only 28.6% of the animals in this group remained (71.4% efficacy; P <0.001). Challenge of the *MBP-FlaA* + LT_{R192G} group with 8×10^{8} bacteria showed a significant reduction in colonization by day 4 (P <0.05) and a drop in bacterial counts throughout the course of the

experiment (Fig. 3C(closed circles). By day 6 the *MBP-FlaA* + LT_{R192G} vaccine resulted in 78% efficacy against colonization (P 0.001), and by day 7 no campylobacters could be detected in the stools under the sampling conditions used (P <0.001).

The data presented here indicate that *MBP-FlaA*, when adjuvanted with LT_{R192G} is capable of eliciting a protective immune response against a heterologous strain of campylobacter as measured in two mouse models involving oral and nasal challenge. At the highest dose (50 micrograms MBP-FlaA + 5 micrograms LT_{R192G}) the vaccine showed 81% protective efficacy against disease and 84% efficacy against colonization of the intestine in the mouse intranasal challenge model (9). In this model immunization with live 81-176, followed by a second infection with the same strain, resulted in 71% efficacy against disease and 91% efficacy against colonization (9). Although the mouse intranasal model uses an unnatural route of infection, it is the only mouse model for *campylobacter* which consistently results in disease symptoms, generally pneumonia and bacteremia (9). Intestinal colonization presumably occurs in this model when the mice swallow some portion of the infecting bacteria. To more directly measure the protection against colonization, we also challenged mice which had undergone the same immunization regimen (50 micrograms *MBP-FlaA* + LT_{R192G}) with different oral doses of 81-176. The results showed that when challenged with 8×10^8 bacteria, there was a reduction in colonization as early as 3 days after infection and that no *campylobacters* could be detected in stools 7 days post-feeding.

Flagella are a key virulence determinant of *Campylobacter* spp. since motility is essential, for establishment of colonization in the mucus lining of the gastrointestinal tract (25, 26, 27,

28). Moreover, flagellin is an immunodominant antigen recognized during infection (16, 17,11,18), and it has been suggested that development of antibodies against flagellin correlates with development of protection (29, 11, 18). The observation that feeding of one strain of campylobacter protects against disease from the homologous, but not heterologous strains, (10) is consistent with the idea that the major protective antigen shows variation among strains. Although there is no flagellar serotyping scheme for campylobacters comparable to the H antigen typing scheme of the *Enterobacteriaceae*, there is serological diversity among campylobacter flagellins (34, 35, 7). In *Salmonella* and *E. coli* it has been demonstrated that the amino and carboxy ends of flagellins are involved in transport of the monomer and assembly into the filament, and these regions are highly conserved among serotypes. The central region of the flagellin protein, which lacks functional constraints, is the antigenically diverse region responsible for H serospecificity, and is also the region which is surface exposed in the flagellar filament. Based on comparison of DNA sequence analyses of flagellin genes from several strains of *C. jejuni*, including that of 81-176 reported here, and one strain of *C. coli* (4,30, 8, 3, 18, 32), the overall structure of campylobacter flagellins appears similar to those of the enterics. Thus, the amino and carboxy terminal regions are highly conserved among campylobacter flagellins, and the central regions are more variable (36). Moreover, Power et al. (7) have shown that antibodies to the amino and carboxy regions are not surface exposed in the flagella filament of campylobacter. The only antibodies found in that study to be surface exposed in the filament were those which recognize a glycosyl posttranslational modification (33, 37, 38). These modifications alter the

apparent M_r of flagellins on SDS-PAGE gels. For example, the masses of the flagellins of VC167 and 81-176 are predicted to differ by only 207, but their apparent difference on SDS-PAGE is greater (see Fig. 1). Moreover, Alm et al. (39) showed that the apparent M_r of flagellin can vary when expressed in different *campylobacter* hosts. The presence of a carbohydrate moiety on a bacterial flagellin is highly unusual and has been shown to confer serospecificity to the flagellin (33). Thus, antisera which recognize the posttranslational modifications on the flagellar filament of VC167 (Lior 8) also react with flagellins of other strains of Lior 8, but not strains of other Lior serogroups (39). Although flagellin is not the serodeterminant of Lior 8 (i.e. non-flagellated mutants of Lior 8 strains still serotype), flagellins appear to be conserved antigenically within the serogroup. Moreover, more recent studies have suggested that glycosyl modifications on flagellin, as well as other campylobacter proteins, are immunodominant (38).

One would expect that any protective epitopes would be surface exposed on the flagellar filament. The role of these surface exposed posttranslational modifications on protection has been addressed in only one study using the removable intestinal tie adult rabbit diarrhea (RITARD) model. In this model protection against colonization appeared to be limited to strains of the same Lior serotype. In other words, immunization by feeding with VC167 protected rabbits against subsequent colonization following RITARD challenge with the homologous strain, as well as two other *C. jejuni* strains of the Lior 8 serogroup, but not against strains of other serogroups (37). A site-specific mutant defective in a gene required for biosynthesis of the posttranslational modification in *VC167* was capable of protecting against a challenge of wildtype *VC167*, but not

the other *C. jejuni* Lior 8 strains, suggesting that the posttranslational modifications are responsible for this Lior 8 serospecific protection. Given this data, one would not expect that recombinant flagellin that lacked the posttranslational modifications, which are encoded by other *campylobacter* genes, would be protective, but the data presented here suggest otherwise.

In this regard, it is interesting that antibodies generated during natural infection in ferrets by either 81-176 or VC167 appeared to react more strongly to glycosylated flagellins isolated from *Campylobacter* spp. than to unglycosylated, recombinant flagellins isolated from *E. coli.* Similar analysis using serum from a human volunteer who had been infected with 81-176 (15) also suggested a stronger immune response to native flagellin than recombinant flagellin. This is documented in (40) and is incorporated by reference. Although the recombinant constuction used contains a truncated FlaA, this region was selected based on its high immunogenicity in a mimeotope mapping study (7). Thus, the lack of immune response to this region with antisera from experimentally infected humans and animals was surprising, and suggests that during natural gastrointestinal infection the immunodominant epitopes are those of the posttranslational modifications rather than the primary amino acids. Immunization with the recombinant fusion protein lacking these posttranslational modifications may lead to antibody production against epitopes which are less immunogenic in the native molecule due to differences in folding and/or masking by the carbohydrate moiety, but are, nonetheless, capable of eliciting a protective immune response. We are currently further evaluating this recombinant flagellin as a vaccine in a ferret diarrheal disease model (21, 22).

Table 1. Resistance to *C. jejuni* 81-176 challenge following vaccination with recombinant FlaA with or without LT.

Dose of MBP-FLaA	LT	n	Disease Index	% Efficacy	Fecal Excretion (day 7) % Colonization	% Efficacy
none	-	13	0.924 +/- 0.214	0	82	0
none	+	11	0.859 +/- 0.297	7	67	18.2
3 ug	-	7	0.771 +/- 0.373	16.6	100	0
6 ug	-	7	0.771 +/- 0.281	16.6	100	0
12 ug	-	7	0.743 +/- 0.433	18.1	100	0
25 ug	-	12	0.483 +/- 0.410	44.1	92	0
50 ug	-	7	0.371 +/- 0.511	55.3	43	47.6
3 ug	+	6	0.793 +/- 0.365	14.1	60	26.8
6 ug	+	7	0.429 +/- 0.378	53.6	100	0
12 ug	+	6	0.667 +/- 0.391	27.8	83.0	0
25 ug	+	12	0.333 +/- 0.445	64.0	77.0	61.0
50 ug	+	8	0.175 +/- 0.244	81.1	13.0	84.0

Table 2. Serum IgG responses measured by ELISA of ferrets infected with *Campylobacter* spp. to campylobacter flagellins and MBP-FlaA.*

	Number (%) of animals responding to:		
Infecting Strain	VC167 flagellin	81-176 flagellin	MBP-FlaA
81-176	8/8 (100%)	8/8 (100%)	3/8 (37.5%)
VC167 T2	6/8 (75%)	7/8 (87.5%)	3/8 (37.5%)

*Animals were infected with between 10^9-10^{10} cells of the indicated strains and serum samples were taken 1 week after infection. An animal was considered to respond to the antigen if there was a greater than 4-fold increase in titer compared to the pre-immune sera.

Table 3. Immunogenicity and efficacy of MBP-FlaA given with and without adjuvant in the mouse intranasal model.

Immunization Regimen			Immunogenicity		Efficacy at day 7			
			geometric mean titer #		Disease Symptoms		Fecal Excretion (%)	
µg MBP-FlaA	LT_{R192G}	n	Lavage IgA	Serum IgG	Illness Index	%Efficacy	%Colonized	%Efficacy
-	-	13	0.9 ± 0	5.7 ± 1.2	0.92 ± 0.21	NA	82*	NA
-	+	11	0.9 ± 0	4.3 ± 1.3	0.86 ± 0.29	7.0	67*	18.3
3	-	7	1.0 ± 0.3	9.9 ± 0.8^{a}	0.77 ± 0.37	16.6	100	0
6	-	7	1.3 ± 0.7	10.6 ± 1.2^{a}	0.77 ± 0.28	16.6	100	0
12	-	7	1.4 ± 0.5	10.2 ± 1.6^{a}	0.74 ± 0.43	18.1	100	0
25	-	12	3.5 ± 1.0^{a}	11.9 ± 1.9^{a}	0.48 ± 0.41	44.1	92	0
50	-	7	5.5 ± 1.1^{a}	14.0 ± 1.5^{a}	0.37 ± 0.51	55.3	43	47.6
3	+	6	$3.5 \pm 0.8^{a,c}$	$13.2 \pm 0.6^{a,c}$	0.79 ± 0.37	14.1	60**	26.8
6	+	7	$3.4 \pm 0.8^{a,c}$	$13.1 \pm 0.5^{a,c}$	0.43 ± 0.38^{b}	53.6	100	0
12	+	6	$3.4 \pm 1.3^{b,d}$	$12.8 \pm 1.0^{a,d}$	0.67 ± 0.39	27.8	83	0
25	+	12	$5.7 \pm 1.2^{a,c}$	$14.1 \pm 1.7^{a,d}$	0.33 ± 0.44^{b}	64.0	75	8.5
50	+	8	$6.6 \pm 0.6^{a,d}$	14.8 ± 0.7^{a}	0.17 ± 0.24^{a}	81.1	13^{b}	84.1

geometric mean titer is expressed as natural log transformed values. a, $P<0.001$ compared to animals immunized with PBS; b, $P<0.05$ compared to animals immunized with PBS; c, $P<0.001$ compared to animals immunized with a comparable dose of MBP-FlaA without adjuvant; d, $P<0.05$ compared to animals immunized with a comparable dose of MBP-FlaA without adjuvant. *2 animals died following challenge; **1 animal died following challenge.

REFERENCES

1. Tauxe, R. V. 1992. Epidemiology of *C. jejuni* infections in the U.S. and other industrial nations, pp 9-19. In I. Nachamkin, M.J. Blaser and L.S. Tompkins (eds), *Campylobacter jejuni*: Current status and future trends. American Society for Microbiology, Washington, D.C.

2. Taylor, D.N. 1992. *Campylobacter* infections in developing countries, pp. 20-30. In I. Nachamkin, M. J. Blaser, and L. S. Tompkins (ed.), *Campylobacter jejuni*: Current status and future trends. Amer. Soc. For Microbiology, Washington, D.C.

3. Kuroki, S., t. Haruta, M. Yoshioka, Y. Kobayaski, M. Nukina, and H. Nakanishi. 1991. Guillain-Barre syndrome associated with *Campylobacter* infection. Pediatr. Infect. Dis. 10:149.

4. Guerry, P., R. A. Alm, M. E. Power, S.M. Logan, and T.J. Trust. 1991. The role of two flagellin genes in *Campylobacter* motility. J. Bacteriol. 173:4757.

5. Guerry, P., R.A. Alm, M.E. Power, and T.J. Trust. 1992. Molecular and structural analysis of *Campylobacter* flagellin, p. 267-281. *In*. I. Nachamkin, M.J. Blaser, and L.S. Tompkin (ed.), *Campylobacter jejuni*: *Current Status and Future Trends*. American Society for Microbiology, Washington, D.C.

6. Guerry, P., S.M. Logan, S. Thornton, and T.J.Trust. 1990. Genomic organization and expression of *Campylobacter* flagellin genes. J. Bacteriol. 172:1853.

7. Power, M.E., P. Guerry, W.D. McCubbin, C.M. Kay and T.J. Trust. 1994. Structural and antigenic characteristics of *Campylobacter* *coli* fla A flagellin. J. Bacteriol. 176:3303.

8. Logan, S.M., T.J. Trust, and P. Guerry. 1989. Evidence for postranslational modification and gene duplication of Campylobacter flagellin. J. Bacteriol. 171(6):3031.

9. Baqar,S., A.L. Bourgeois, L.A. Applebee, A.S. Mourad, M.T. Kleinosky, Z. Mohran, and J.R. Murphy. 1996. Murine intranasal challenge model for the study of *Campylobacter* pathogenesis and immunity. Inf. Imm. 64:4933.

10. Black, R.E., M. M. Levine, M.L. Clements, T.P. Hughes and M.J. Blaser. 1988. Experimental Campylobacter jejuni infection in humans. J. Infect Dis. 157: 472-479.

11. Martin, P.M., J. Mathiot, J. Ipero, M. Kirimat, A. J. Georges, and M.C. Georges-Courbot. 1989. Immune response to Campylobacter jejuni and Campylobacter coli in a cohort of children from birth to 2 years of age. Infect. Immun. 57:2542-2546.

12. Lior, H., D. H. Woodward, J.A. Edgar, L.J. Laroche, and P. Gill. 1982. Serotyping of Campylobacter jejuni by side agglutination based on heat-labile antigenic factors. J.Clin. Microbiol. 15: 761-768.

13. Moran, A. P., B.J. Appelmelk, and G.O. Aspinall. 1996. Molecular mimicry of host structures by lipopolysaccharides of Campylobacter and Helicobacter spp.: implications for pathogenesis. J.Endotox Res. 3(6):521-531.

14. Scott, D.A. 1997. Vaccines against Campylobacter jejuni. J. Infect. Dis. 176 (Suppl 2): S183-188

15. Tribble, D. Unpublished data.

16. Blaser, M.J. and D.J. Duncan. 1984. Human serum antibody response to Campylobacter jejuni infection as measured in an enzyme-linked immunoabsorbent assay. Infect. Immun. 44:297-298.

17. Blaser, M.J., J.A. Hopkin, and M.L. Vasil. 1984. Campylobacter jejuni outer membrane proteins are antigenic for humans. Infect. Immun. 43:986-993.

18. Nachamkin, I. And A. M. Hart. 1985. Western blot analysis of the human antibody response to Campylobacter jejuni cellular antigens during gastrointestinal infection. J. Clin. Microbiol. 21:33-38.

19. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.

20. Logan, S.M. and T.J. Trust. 1983. Molecular identification of surface protein antigens of Campylobacter jejuni. J. Bacteriol. 168:739-745.

21. Doig, P., R. Yao, D. H. Burr, P. Guerry and T. J. Trust. 1996. An environmentally regulated pilus-like appendage involved in Campylobacter pathogenesis. Mol. Microbiol. 20(4):885-894.

22. Yao, R., D. H. Burr, and P. Guerry. 1997. CheY-mediated modulation of Campylobacter jejuni virulence. Mol. Microbiol. 23:1021-1032.

23. Baqar, S., A.L. Bourgeois, P.J. Schultheiss, R.I. Walker, D.M. Rollins, R.L. Halberberger, and O.R. Pavlovskis. 1995. Safety and immunogenicity of a prototype oral whole-cell killed Campylobacter vaccine adminstered with a mucosal adjuvant in non-human primates. Vaccine 13:22-28.

24. Yao, R., D. H. Burr, P. Doig, T. J. Trust, H. Niu, and P. Guerry. 1994. Isolation of motile and non-motile insertional mutants of Campylobacter jejuni defective in invasion of eukaryotic cells: the role of flagella in invasion. Mol. Microbiol. 14(5):883-893.

25. Lee, A., J.L. O'Rourke, P. J. Barrington, and T.J. Trust. 1986. Mucus colonization by Campylobacter jejuni: a mouse cecal model. Infect. Immun. 51:536-546.

26. Morooka, T., A. Umeda, and K. Amaka. 1985. Motility as an intestinal colonization factor for Campylobacter jejuni. J. Gen. Microbiol. 131:1973-1980.

27. Pavlovskis, O.R., D. M. Rollins, R. L. Haberberger, Jr., A. E. Green, L. Habash, S. Stroko, and R. I. Walker. 1991. Significance of flagella in colonization resistance of rabbits immunized with Campylobacter spp. Infect. Immun. 59:2259-2264.

28. Wassenaar, T. M., B.A.M. Van der Zeijst, R. Ayling, and D. G. Newell. 1993 Colonization of chicks by motility mutants of Campylobacter jejuni demonstates the importance of flagellin A expression. J. Gen. Microbiol. 139:1171-1175.

29. Butzler, J.P., Y. Glupczynsk, and Y. Goodson. 1992. Campylobacter and Helicobacter infections. Curr. Opin. Infect. Dis. 5:80-87.

30. Khawaja, R., K. Neote, H.L. Bingham, J. L. Penner, and V.L. Chan. 1992. Cloning and sequence analysis of the flagellin gene of Campylobacter jejuni TGH9011. Curr. Microbiol. 24:213-221.

31. Meinersmann, R. J., L.O. Helsel, P. I. Fields, and K.L. Hiett. 1997. Discrimination of Campylobacter jejuni isolates by fla gene sequencing. J. Clin. Microbiol. 35:2810-2814.

32. Nuijten, P. J., F. J. van Asten, W. Gaastra, and B. A. van der Zeijst. 1990. Structural and functional analysis of two Campylobacter jejuni flagellin genes. J. Biol. Chem. 265:17798-17804.

33. Doig, P., N. Kinsella, P. Guerry, and T.J. Trust 1996. Characterization of a posttranslational modification of Campylobacter flagellin: identification of a serospecific glycosyl moiety. Mol. Microbiol. 19(2):379-387.

34. Harris, L.A., S.M. Logan, P. Guerry, and T.J. Trust. 1987. Antigenic variation of *Campylobacter* flagella. J. Bacteriol. 169:5066-5071.

35. Logan, S.M. and T. J. Trust. 1986. Location of epitopes on *Campylobacter jejuni* flagella. J. Bacteriol. 168:739-745.

36. Homma, M., H. Fujita, S. Yamaguchi, and T Iino. 1987. Regions of Salmonella typhimurium flagellin is essential for its polymerization and excretion. J. Bacteriol. 169:291-296.

37. Guerry, P., P. Doig, R.A. Alm, D. H. Burr, N. Kinsiella, and T. J. Trust. 1996. Identification and characterization of genes required for post translational modification of *Campylobacter coli VC167* flagellin.

38. Szymanski, C.M., R. Yao, C. P. Ewing, T. J. Trust and P. Guerry. 1999. Evidence for a system of general protein glycosylation in *Campylobacter jejuni*. Mol. Microbiol. (in press)

39. Alm, R.A., P. Guerry, M.E. Power, and T.J. Trust. 1992. Variation in antigenicity and molecular weight of Campylobacter coli VC167 flagellin in different genetic backgrounds. J. Bacteriol. 174:4230-4238.

40. Lee, L. H., E. Burg, S. Baqar, A. L. Burgeois, D. H. Burr, C. P. Ewing, T. J. Trust and P. Guerry. Evaluation of Truncated Recombinant Flagellin Subunit Vaccine against Campylobacter jejuni. Infect. and Immun. 67:5799-5805.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that, within the scope of the appended claims, the invention may be practiced otherwise than as specifically described. Accordingly, the recombinant construct can be used as a fusion protein with not only MBP, described here, but with other protein expression systems. The recombinant construct can also be expressed in carrier vaccines such as *Salmonella* or *Shigella* attenuated, live vaccines, for use as a bivalent vaccine.